Sephadex®

Gel filtration in theory and practice

Pharmacia Fine Chemicals AB is a subsidiary of Pharmacia AB, Uppsala, Sweden, one of the oldest pharmaceutical companies in Scandinavia. Pharmacia pioneered extensive research work on dextran. The plasma volume expander, Macrodex, and the "flow improver", Rheomacrodex, are well known dextran products in hospitals all over the world. Many fine chemicals are now derived from dextran. The most important one, Sephadex, is used for gel filtration. Pharmacia Fine Chemicals also manufactures ion exchangers derived from dextrans, dextran fractions and derivatives and Ficoll, a polymer of high molecular weight with low intrinsic viscosity.

This booklet is intended as an introduction to gel filtration and also as a reference book for those already working in this field. Special emphasis has therefore been given to the sections dealing with gel filtration technique and practical applications.

Pharmacia Fine Chemicals has also issued introductory booklets on the other products mentioned above. These will be sent upon request. Pharmacia Fine Chemicals will be pleased to answer specific questions concerning applications of these products and to hear about new applications or techniques involving their use. Personal communications or reprints of published work would be welcome.

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WHAT IS SEPHADEX?

Sephadex is a bead-formed, dextran gel. Sephadex is prepared by cross-linking selected dextran fractions with epichlorohydrin. Dextran is an anhydroglucose polymer produced in sucrose-containing solutions by different strains of *Leuconostoc mesenteroides*. Because of the high content of hydroxyl groups in the polysaccharide chains, Sephadex is strongly hydrophilic and thus swells in water and electrolyte solutions. Sephadex is a chromatographic material capable of separating substances according to molecular size. The separation method is most commonly known as *gel filtration* or *gel chromatography*. Sephadex is supplied in the form of minute beads. The bead form has considerable advantages, as it imparts good flow and separation properties to chromatographic materials.

Various types of Sephadex are available, differing in their swelling properties. The degree of swelling is an important characteristic of the gel. Gels in which the matrix is a minor component are used for fractionation of high molecular weight substances, whereas the denser gels are used for separation of low molecular weight compounds.

Sephadex LH-20 is a hydroxypropylated derivative with lipophilic as well as hydrophilic properties and therefore swells in many organic solvents as well as water.

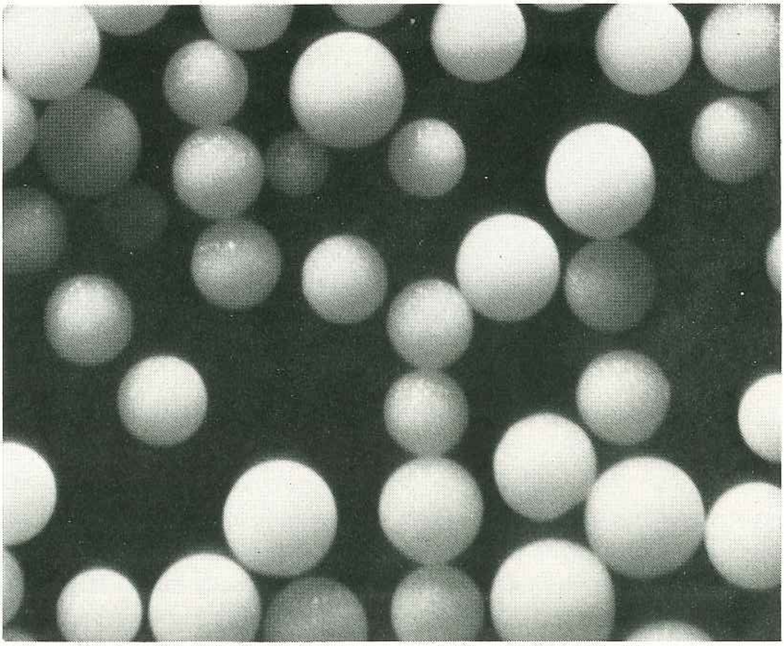


Fig. 1a "Sephadex", a bead polymerized dextran.

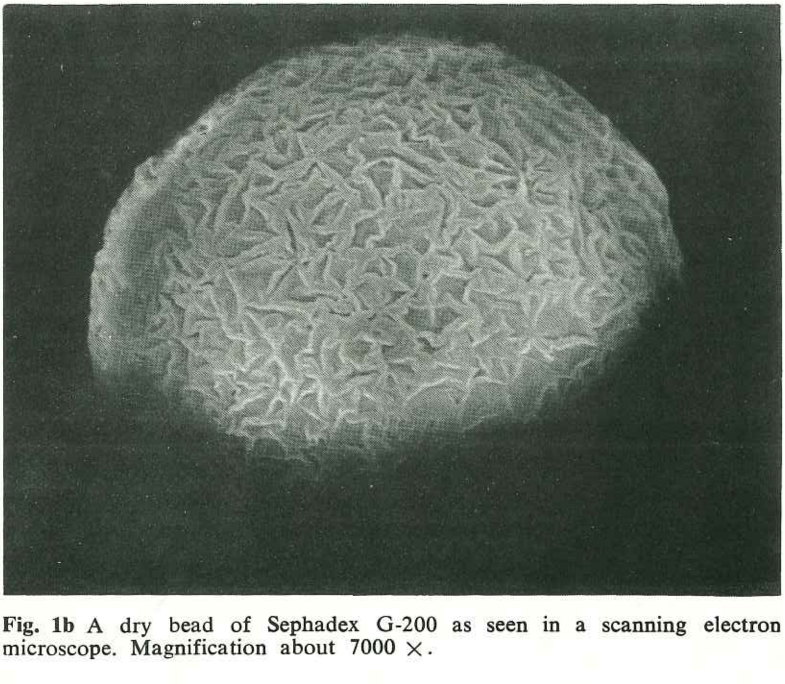


Fig. 1b A dry bead of Sephadex G-200 as seen in a scanning electron microscope. Magnification about 7000 ×.

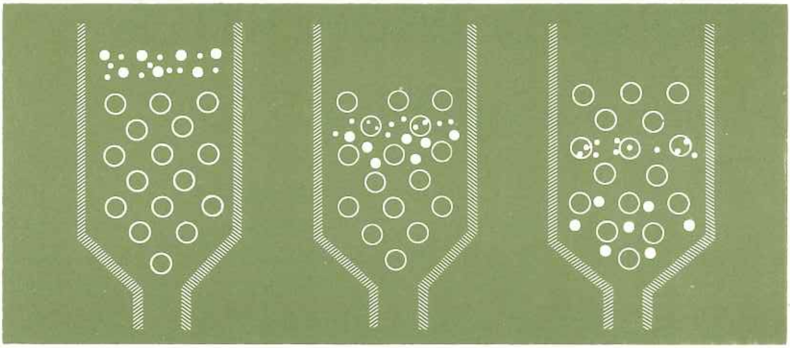
GEL FILTRATION WITH SEPHADEX

Gel filtration first became an established laboratory technique with the introduction of Sephadex in 1959. Since then, gel filtration has been increasingly used both in analytical and in preparative work, as well as on a production scale in the chemical industry.

Before Sephadex was introduced, fractionation and separation of molecules according to size could only be carried out by very time-consuming or expensive methods. Using the appropriate type and grade of Sephadex, such separations can now be performed rapidly and simply. Sephadex is particularly useful for chromatography of substances of biological origin, which are often very labile.

A gel filtration experiment can be schematically described in the following way.

Molecules larger than the largest pores of the swollen Sephadex, i.e. above the exclusion limit, cannot penetrate the gel particles and therefore they pass through the bed in the liquid phase outside the particles. They are thus eluted first. Smaller molecules, however, penetrate the gel particles to a varying extent depending on their size and shape. Molecules are therefore eluted from a Sephadex bed in order of decreasing molecular size.



Three stages of a simple separation on a Sephadex bed are diagrammatically illustrated in the figure. The large dots represent large molecules, the small dots, small molecules and the open circles Sephadex particles. As the molecules pass through the bed at different rates, they emerge from the outlet of the column separated from each other. The zones eluted are somewhat wider than the sample applied, but by the use of suitable experimental conditions this zone broadening can be limited. When all the molecules have been eluted from the Sephadex bed, the column is ready for another experiment. Regeneration is thus unnecessary. This is one of the advantages of gel filtration: the same column can generally be used for a large number of experiments.

SEPHADEX PROPERTIES

Physical properties

The properties of Sephadex of the G-series are given in table I. These Sephadex types differ in degree of cross-linkage and thus in swelling properties. To characterize the gels with respect to their swelling ability a so called water regain value has been used. It represents the amount of water imbibed by the gel grains on swelling and does not include the water between the grains. The type numbers refer to the water regain value of the gels. Sephadex G-10 thus has a water regain value of about 1 and Sephadex G-200 has a water regain value of about 20 ml/g dry gel.

Sephadex G-types also swell in dimethylsulphoxide, formamide and glycol. Mixtures of water and the lower alcohols may also be used. In addition to these solvents, Sephadex G-10 and G-15 swell in dimethylformamide. It should, however, be noted that the solvent regain in solvents other than water is not equivalent to the water regain. Sephadex LH-20, obtained by hydroxypropylation of Sephadex G-25, has the property of swelling in many polar organic solvents as well as in water (table 2).

Sephadex is available in different particle size grades. The superfine grade is intended for column chromatography requiring very high resolution and for thin-layer chromatography. The fine grade is recommended for preparative purposes, where the extremely good resolution that can be achieved with the superfine grade is not required, but where the flow rate is of greater importance. The coarse and medium grades are intended for preparative chromatographic processes where a high flow rate at a low operating pressure is essential. In addition the coarse grade is suitable for batch procedures.

Table I

Sephadex type		Dry particle diameter μ	Fractionation range (MW)		Bed volume ml/g dry Sephadex
			Peptides and globular proteins	Dextrans	
Sephadex G-10		40—120	— 700	— 700	2—3
Sephadex G-15		40—120	— 1,500	— 1,500	2.5—3.5
Sephadex G-25	Coarse	100—300	1,000— 5,000	100— 5,000	4—6
	Medium	50—150			
	Fine	20— 80			
	Superfine	10— 40			
Sephadex G-50	Coarse	100—300	1,500— 30,000	500— 10,000	9—11
	Medium	50—150			
	Fine	20— 80			
	Superfine	10— 40			
Sephadex G-75		40—120	3,000— 70,000	1,000— 50,000	12—15
	Superfine	10— 40			
Sephadex G-100		40—120	4,000—150,000	1,000—100,000	15—20
	Superfine	10— 40			
Sephadex G-150		40—120	5,000—400,000	1,000—150,000	20—30
	Superfine	10— 40			18—22
Sephadex G-200		40—120	5,000—800,000	1,000—200,000	30—40
	Superfine	10— 40			20—25

Table II

Solvent	Appr. bed volume ml/g dry gel	Solvent	Appr. bed volume ml/g dry gel
Dimethyl sulphoxide	4.4—4.6	Methylene dichloride	3.6—3.9
Pyridine	4.2—4.4	Butanol	3.5—3.8
Water	4.0—4.4	Isopropanol	3.3—3.6
Dimethylformamide	4.0—4.4	Tetrahydrofuran	3.3—3.6
Methanol	3.9—4.3	Dioxane	3.2—3.5
Ethylene dichloride	3.8—4.1	Acetone	2.4—2.6
Chloroform[1]	3.8—4.1	Acetonitrile[3]	2.2—2.4
Propanol	3.7—4.0	Carbon tetrachloride	1.8—2.2
Ethanol[2]	3.6—3.9	Benzene	1.6—2.0
Iso-butanol	3.6—3.9	Ethyl acetate	1.6—1.8
Formamide	3.6—3.9	Toluene	1.5—1.6

[1] Containing 1 % ethanol

[2] Containing 1 % benzene

[3] Solvents giving a bed volume of less than about 2.5 ml/g dry gel are generally not useful

Fractionation ranges and chromatographic properties

The choice of the appropriate Sephadex type depends on the molecular size and the chemical properties of the substances to be separated.

Each Sephadex type fractionates within a particular molecular weight range, determined by the degree of swelling of the gel. Molecules of a molecular weight above the upper limit of this range—the exclusion limit—are totally excluded from the gel. Molecules of a molecular weight below the fractionation range are usually eluted at an elution volume approximately equal to the total bed volume.

Table I gives the molecular weight ranges within which fractionation can be achieved by different Sephadex types. The superfine grades of Sephadex G-75 to G-200 swell slightly less than the normal types and consequently have somewhat lower exclusion limits. The correlation between K_{av} (see page 56) and molecular weight (logarithmic scale) of proteins on various Sephadex types is given in fig. 2.

A gel should be chosen so that separation occurs in the linear part of the K_{av} versus molecular weight diagram. With this restriction the exclusion limit should fall just above the molecular weight of the substance of interest. Zones corresponding to low K_{av} values are narrower than those eluted near the total bed volume. Attainment of equilibrium between the mobile and gel phases is less critical for substances which cannot diffuse to any great extent into the gel than it is for substances for which the whole inner volume of the gel is available. Non-equilibrium and thus zone broadening is therefore more likely to occur in the latter part of the elution diagram.

Certain molecular species deviate strongly from the general elution behaviour described above. Notably, aromatic substances (homocyclic or heterocyclic) show greater affinity for Sephadex (gel phase) than for the

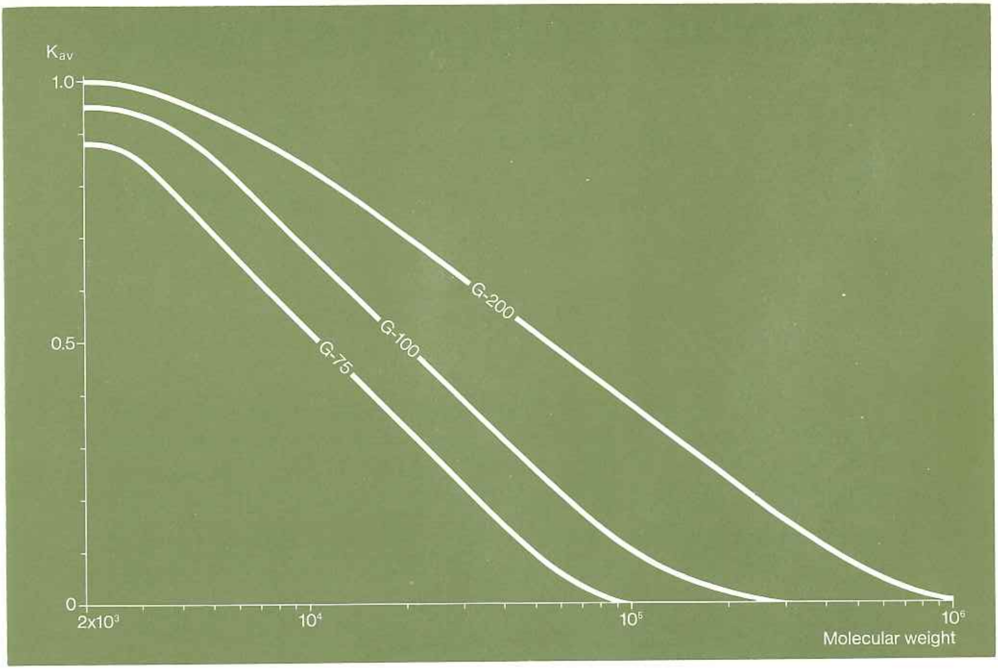


Fig. 2 Relationship between elution behaviour and molecular properties: K_{av} versus molecular weight (on a logarithmic scale) for globular proteins. (By kind permission of Dr. P. Andrews.)

aqueous phase, and are thus usually retarded to varying extents compared with non-aromatic substances of similar size. In addition to the affinity of aromatic substances for the Sephadex matrix in aqueous solutions, some special affinities have been observed in work with Sephadex LH-20 in organic solvents. Thus, solutes containing hydroxyl and carboxyl groups are retarded more than would be expected from their molecular size when they are chromatographed on Sephadex LH-20 in chloroform.

The Sephadex matrix contains a small number of carboxyl groups, which at low ionic strength cause interaction between charged solutes and the matrix. Thus, positively charged substances may be retarded, resulting in tailing of the elution curve, while negatively charged substances are excluded from the gel phase. These effects can be eliminated by the use of solvents with an ionic strength exceeding 0.02.

Chemical stability of Sephadex

Sephadex is insoluble in all solvents (unless it is chemically degraded). It is stable in water, salt solutions, organic solvents, alkaline and weakly acidic solutions. In strong acids the glycosidic linkages in the gel matrix are hydrolysed. However, Sephadex can be exposed to 0.1 M HCl for 1—2 hours without noticeable effects, and in 0.02 M HCl Sephadex is still unaffected after 6 months. Prolonged exposure to oxidizing agents will affect the gel and should be avoided.

Sephadex can be sterilized in the wet state at neutral pH or dry by autoclaving for 30 minutes at 120°C without any changes in the properties of the gel. If dry Sephadex is heated to more than 120°C, it will start to caramelize.

APPLICATIONS

Sephadex for analytical purposes

Analysis of multicomponent samples

In gel filtration quantitative yields are obtained as there is no irreversible retention of small amounts of substance on the columns. This makes Sephadex very useful for analytical purposes.

The chromatographic experiments can be made quantitative by recording continuously some physical variable in the effluent (e.g. light absorption, optical activity, refractive index). The effluent can also be collected in fractions and the amounts of substance in them evaluated by physical, chemical or biological tests.

Gel filtration can often be used for analysis where few or no other methods are available. It is thus used for analysis of protein mixtures and fluids of biological origin.

A very common application of gel filtration is the analysis of mixtures of molecules of different molecular weight. An example of this application is given in fig 3, showing the separation of raffinose, maltose and glucose from each other and from KCl on Sephadex G-15. Fig. 4 shows the elution pattern of an artificial mixture of glycerides on Sephadex LH-20 in chloroform. Glycerol triesters are eluted in order of decreasing molecular weight but dipalmitin and monostearin are retarded. It should be stressed that separation can be obtained without differences in molecular weight if the substances have different sterical properties or if there is a direct interaction between the substances and the gel matrix. Very often molecules of similar molecular weight can be separated and analysed on Sephadex beds. For instance gel filtration is often used to determine the contents of aromatic substances in a mixture, as aromatic substances are usually retarded on a column compared with aliphatic substances of similar molecular weight (see page 10) 2044 2049*

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*Number refers to the Literature Reference List

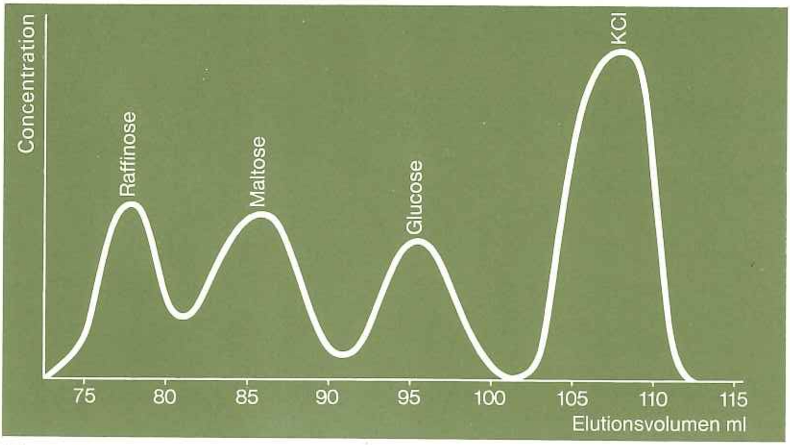

Fig. 3 Desalting and separation of raffinose, maltose and glucose on Sephadex G-15.
Sample volume: 0.5 ml. Bed dimensions: 1.4×100 cm. Flow rate: 6 ml/h. Eluant: 0.025 M phosphate buffer, pH 7.
Virtually the same result is obtained with distilled water as with phosphate buffer eluant.

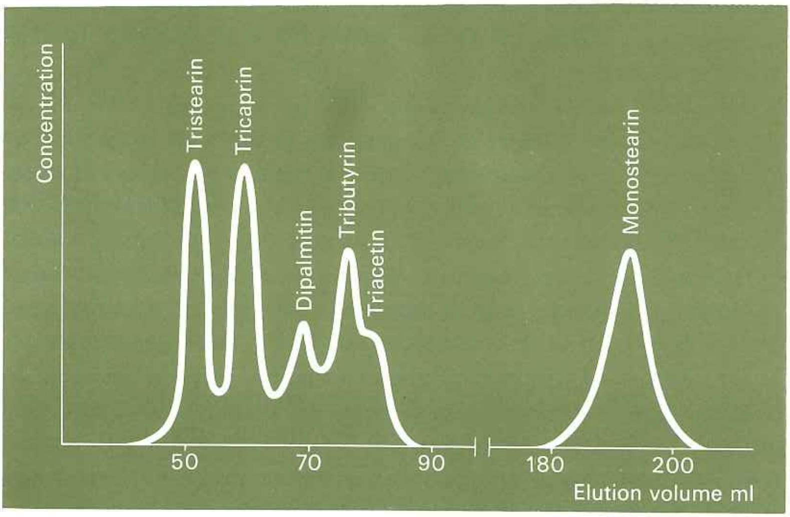

Fig. 4 Separation of an artificial mixture of glycerides on Sephadex LH-20 in chloroform. (Joustra, M. K., Protides of the Biological Fluids 14 (1966) 533—541. Reproduced by kind permission of the Author and the Publisher.)

Determination of molecular weights

One of the most striking properties of Sephadex gels as chromatographic materials is their capacity for separating substances according to molecular size. For proteins, extensive investigations have shown that the elution volumes of globular proteins are largely determined by their molecular weight. Over a considerable range, the elution volume is approximately a linear function of the logarithm of the molecular weight. Gel filtration can also be used for determination of molecular weights of peptides and of other macromolecules than proteins. As the relationship between molecular weight and elution volume is different for different types of molecules, a separate calibration curve should be determined for each type. References to molecular weight determination are given in the section "Selected References" (see page 60).

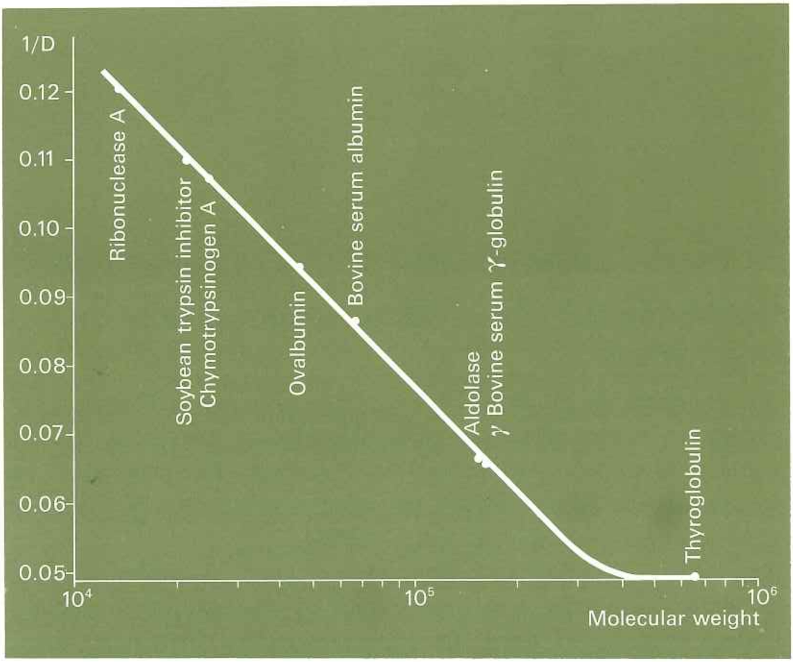

Fig. 5 Relationship between the reciprocal of the migration distance **(1/D)** and the molecular weight of proteins separated by thin-layer gel filtration on Sephadex G-150 Superfine.
Thin-layer plate: 20 × 30 cm. Layer thickness: 0.5 mm. Time: 7 hrs 40 min. Angle: 15°. The proteins were transferred from the gel layer to a Whatman 3 MM paper which was stained in 0.1 % bromophenol blue methanol/glacial acetic acid (9:1, v/v). Migration distances were measured on the paper replica after rinsing with 5 % acetic acid.

With this method the molecular weight of biologically active substances such as enzymes can be determined even in crude preparations. The enzyme activity in the column effluent or on the thin-layer is measured and thus the elution volume or the migration rate can be determined. From the calibration curve, produced by chromatography of proteins of known molecular weight, the molecular weight of the substance can then be estimated. This is the only method of estimating molecular weights of proteins which does not require very extensive purification. For the determination of molecular weights of small samples of substances, thin-layer gel filtration may be used. The calibration curves in this case are very similar to those obtained in column experiments. Fig. 2 illustrates the correlation between elution volume and molecular weight for proteins in column chromatography on Sephadex G-200, while fig. 5 gives the correlation between migration distance and molecular weight in thin-layer gel filtraton on Sephadex G-150 Superfine.

Determination of molecular weight distribution of polymers

The molecular weight distribution is very important for characterization of natural and synthetic polymers. Distribution analysis by classical methods is difficult and tedious as it involves fractionation of the macromolecules by precipitation and determination of molecular weight and amount of substance in every fraction.

The ability of Sephadex columns to fractionate molecules according to molecular weight offers an improved method for distribution analysis of many polymers. The elution curve can be recorded continuously or determined by investigating individual fractions. It is unnecessary to determine molecular weights of the polymers in the effluent fractions of individual experiments, as the chromatographic behaviour of the columns is very reproducible. A calibration curve once determined for the column can be applied to a large number of runs. The precision of this method of distribution analysis is very high due to the good resolution obtained with Sephadex. The method has been used for the determination of molecular weight distributions of dextrans, polyvinylpyrrolidones, gelatine preparations and other water-soluble polymers. References to molecular weight distribution analysis are given in the section "Selected References" (see page 60). In the low molecular weight range this method of distribution analysis can be extended to polymers only soluble in organic solvents by the use of Sephadex LH-20.

Determination of equilibrium constants

In some instances, gel filtration has proved to be a valuable technique for the determination of chemical equilibria. In the case of slow reactions, where the reactants and the products can be separated on a Sephadex column, these substances can be quantitatively determined in the effluent, thereby establishing the position of the equilibrium. [97, 500, 524]

Gel filtration can also be used to determine the position of equilibria of complex formation where the reactions leading to the equilibria are fast. In this case, one of the reactants is chromatographed in an eluant containing the other reactant. From the elution curve of the reactants the complex formation can be studied. This method is of great value in the study of protein binding of low molecular weight substances such as drugs [124, 261, 322, 567] and can be extended to the study of, for instance, competition of two molecules for the same sites. [1064]

Gel filtration has also been applied to the estimation of reaction rates. [218, 838]

Preparative applications

Desalting

Desalting was one of the first applications of gel filtration to be described, and it is still one of the most important.

In this context the term "desalting" refers not only to the removal of salts, but also to the removal of other low molecular weight compounds from solutions of macromolecules. Thus, according to this definition, the production of lactose-free milk is considered as a desalting process. In desalting, the high molecular weight substances to be desalted usually move with the void volume, i.e. they are totally excluded from the gel while the low molecular weight components are approximately equally distributed between the stationary phase and the mobile phase. As the separation between the compounds of high and low molecular weight is very good, large sample volumes may be used. This is illustrated in fig. 6, which gives the elution curves from a desalting column at different sample size. Sample volumes up to approximately 30 % of the total bed volume may be used. [47]

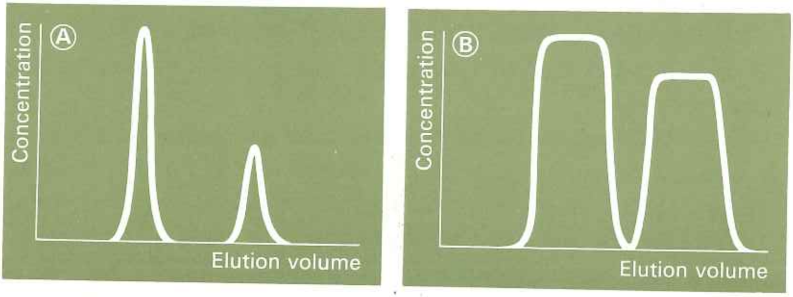

Fig. 6 Elution curves from two experiments on the separation of albumin and sodium chloride.
Bed dimensions: 5.0×50.8 cm (Column K 50/60). Eluant: distilled water. Flow rate: 5 ml/min. Sample volume in A: 30 ml. Sample volume in B: 300 ml. In experiment A the albumin was diluted 5-fold whereas in experiment B 1 ½-fold dilution occurred with the sample volume increased to 30 % of the bed volume.

This desalting method has numerous applications. It is a faster and more efficient technique than dialysis and it is therefore often used for desalting labile substances of biological origin. After modification of macromolecules, excess reagent may be removed by gel filtration. Thus, in immunofluorescence experiments, gel filtration is used as a standard method for separation of the labelled proteins from unreacted fluorescent reagents.[30, 32, 42, 44, 135, 136, 206, 695, 1852] In the phenol extraction method of nucleic acid preparation, aqueous solutions of the nucleic acids containing large amounts of phenol are obtained. Gel filtration may be used to separate the nucleic acids from the phenol.[203, 815] In the preparation of samples for electrophoresis and ion exchange chromatography, buffers may be conveniently exchanged by gel filtration.[47]

In desalting, the conventional column technique is limited by the fact that the viscosity of the sample relative to the eluant should not exceed 2—3 (see page 42). To desalt concentrated solutions of high viscosity, the use of a centrifuge technique is advantageous. This technique will be described under the heading "Industrial applications".

Isolation and fractionation of substances

In desalting, molecules of high molecular weight, which move with the void volume, are separated from low molecular weight material. In many cases, this simple fractionation is insufficient. The separation method must be made more specific for the substances to be purified. The use of the most suitable gel type, long Sephadex beds and collection of small fractions allow separation of molecules differing very little in size and properties. An example of such a separation is given in fig. 7, showing the separation of serum proteins on a column of Sephadex G-200. Haemoglobin and ceruloplasmin were also added to the serum sample. As can be seen from the figure, fractionation of the proteins is obtained. Although serum proteins cannot be completely separated in this way, very good enrichment of individual proteins results.

To achieve an efficient fractionation of a sample, the gel type should be chosen so that the substances of interest fall within its fractionation range. When working with very complex mixtures, e.g. biological fluids, substances often cannot be isolated in a single operation. In such cases gel filtration on different Sephadex types can be used in conjunction with other separation methods. These other separation methods should be based on parameters other than molecular size. For instance ion exchange chromatography or preparative electrophoresis may be used.[101] Fig. 8 illustrates the isolation of transferrin and albumin from plasma by gel filtration followed by ion exchange chromatography on DEAE-Sephadex, a weak anion exchanger based on Sephadex.

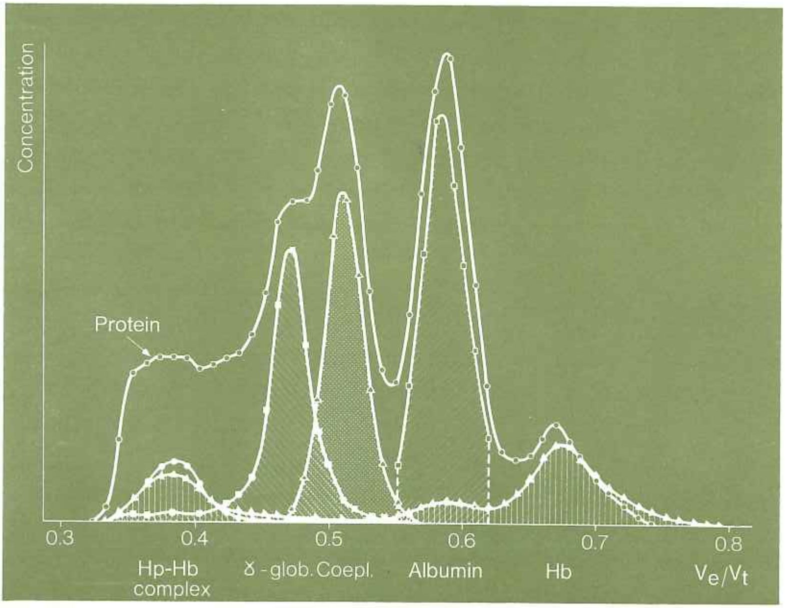

Fig. 7 Fractionation of human serum proteins and haemoglobin on Sephadex G-200.
Bed dimensions: 4.2×73.5 cm. Flow rate: 10—20 ml/h. Eluant: 0.1 M tris-HCl buffer, pH 8.0 and 1 M NaCl. (Killander, J., Biochim. biophys. Acta 93 (1964) 1—14. Reproduced by kind permission of the Author and the Editor.)

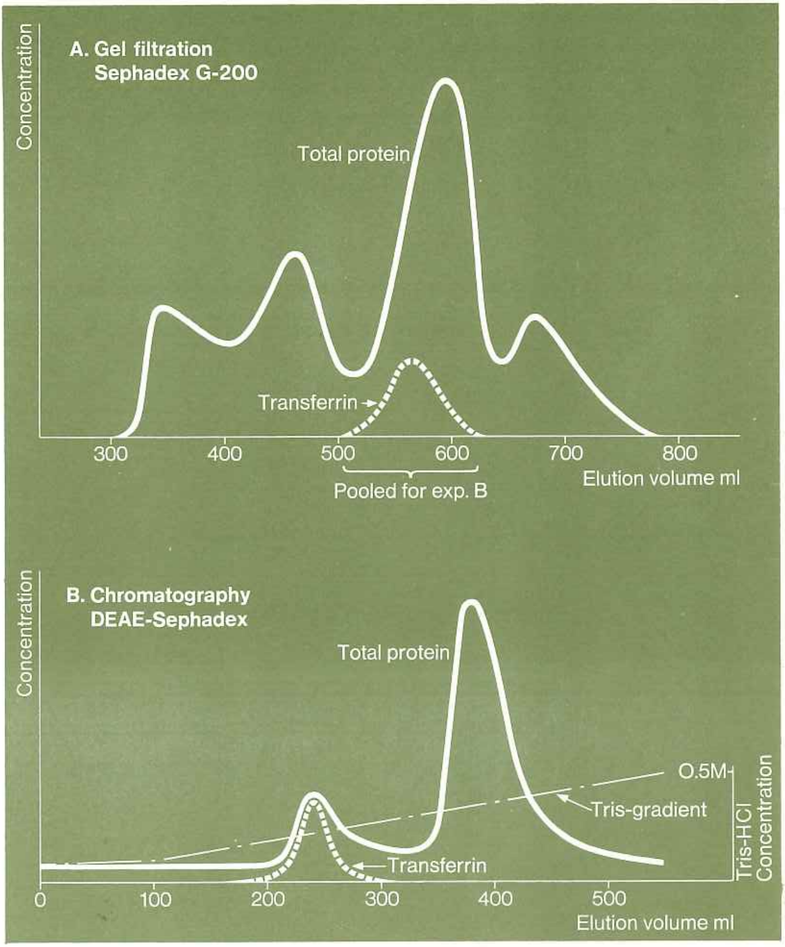

Fig. 8 Purification of transferrin from 6.5 ml lipoprotein-depleted human sera labelled with ^{59}Fe by gel filtration on Sephadex G-200 and chromatography on DEAE-Sephadex A-50.
The gel filtration experiment was performed under teh following conditions. Bed dimensions: 4.2×72.5 cm. Flow rate: 20 ml/h. Eluant: 0.1 M tris-HCl buffer, pH 8.0 and 1 M NaCl. The protein content was detected from light absorption at 254 mμ. The transferrin content was determined from its radioactivity.
The ion exchange experiment was performed on a 2.2×25 cm bed of DEAE-Sephadex A-50. Gradient elution: 0.05 M—0.5 M tris-HCl buffer, pH 8.0. Sample: the third peak from the gel filtration experiment. The transferrin peak from the ion exchanger experiment was homogeneous in starch-gel electrophoresis and immunoelectrophoresis. (Reproduced by kind premission of Dr. J. Killander.)

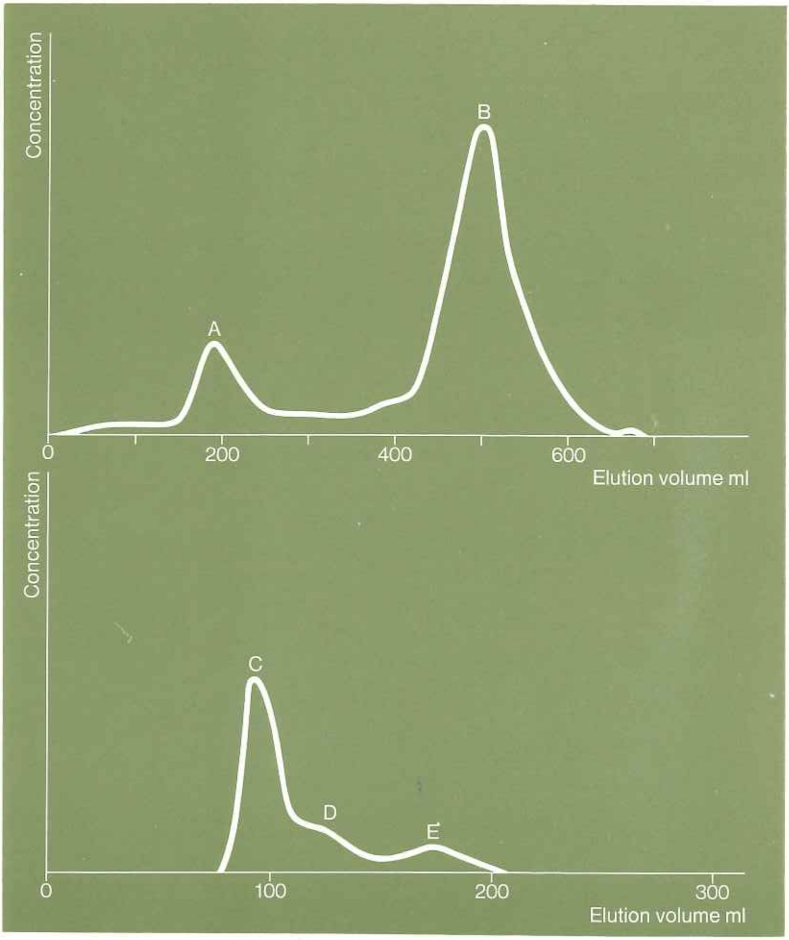

Fig. 9 Purification of oxytocin and vasopressin by gel filtration under varying conditions. In the first experiment, the extract was run on a Sephadex G-25 bed in 0.2 M pyridine-acetic acid buffer, pH 5.9. The oxytocin and vasopressin are eluted in the void volume (peak A). In the second experiment the same experimental conditions were used except that the eluant was 1 M formic acid. The oxytocin and vasopressin were obtained in peak E. (Lindner, E. B., Elmquist, A., Porath, J., Nature 184 (1959) 1565—1566. Reproduced by kind permission of the Authors and the Editor.)

In many instances very good purification can be obtained by successive gel filtration experiments under different conditions. Fig. 9 illustrates the purification of vasopressin and oxytocin.[4] The pituitary extract used as a starting material contains the peptide hormones complexed with a protein, neurophysin. In the first gel filtration step, the hormones move with the void volume together with the proteins. In the next experiment the pH is changed so as to dissociate the hormone protein complex. The peptide hormones are now retarded on the bed. This procedure gives very high purification of the hormones.

Another example is the purification of yeast pyruvate kinase. This enzyme binds to Blue Dextran in 5mM $MgSO_4$, 3 mM 2-mercaptoethanol. The complex is eluted in the void volume on Sephadex G-200. In 30 % $(NH_4)_2SO_4$ the complex is split and the purified enzyme can be separated from the Blue dextran by re-chromatography on Sephadex G-200.[2120]

Industrial applications

Sephadex gel filtration can conveniently be applied to isolation and purification processes on a large scale.[2164] In many biochemical and pharmaceutical industries extensive development work is in progress to replace time-consuming and inefficient procedures by gel filtration. In these applications, the favourable mechanical properties of the dense gels are of great advantage. No regeneration is necessary and very concentrated solutions can be treated.

The Sephamatic® System, comprising equipment for large scale use of Sephadex G-10, G-15 and G-25 allows the use of bed volumes up to 2500 litres.

For large scale work with Sephadex G-75—G-200, specially designed columns are necessary.

The Sephamatic® System is described in a separate booklet: "Industrial gel filtration with the Sephamatic System".

Desalting

Desalting on Sephadex is a very efficient process, as large columns can be used at a high flow rate. For industrial desalting, Sephadex G-25 Coarse is recommended on account of its good flow properties. Separation of serum albumin and ammonium sulphate is a commonly performed operation in laboratories and production plants. Below is an outline of such a separation performed by gel filtration in a Sephamatic gel filter GF 04—10 (diameter, 40 cm; height, 100 cm) packed with Sephadex G-25 Coarse.

Case I is an almost complete desalting achieved in a practical run whilst case II is a computer calculation for a less complete desalting process.

Results with Sephamatic GF 04—10 packed with Sephadex G-25 Coarse.

Important operation data	Case I Practical run (almost complete desalting)	Case II Computer calculation (a less complete desalting operation)
Ammonium sulphate concentration in the albumin* solution:		
before treatment	24 %	24 %
after treatment	0.01 %	0.2 %
Amount of albumin solution:		
before treatment	17 l	31 l
after treatment	40 l	45 l
Time of treatment	48 min	27 min
Loss of albumin	1 %	1.5 %
Input of albumin solution in continuous operation	21 l/h	70 l/h
Estimated process costs (capital costs included)	$ 4/h	$ 4/h

*Calf serum albumin was used as a test substance

The above figures are valid not only for serum albumin and ammonium sulphate, but for almost any protein and any salt.

By varying the equipment size and process conditions, the input/output figures and the degree of desalting can be varied within wide limits. The biggest Sephamatic unit can treat up to 1000 litres of charge solution per hour.

To obtain a continuous process, an automated multi-column arrangement may be used. In this type of setup, a number of columns are run with a phase difference between the cycles of the individual columns. This arrangement allows feed and elution pumps to be run uninterruptedly. It is also usually easier to pack and to operate a number of small columns than to use a larger one of the same capacity.

Serum fractionation

The column K 215/100 can be used for this application.

Gel type		Sephadex G-100
Bed dimensions	diameter:	20 cm
	height:	100 cm
	volume:	32 l
Charge solution	composition:	horse serum
	volume:	0.6 l/cycle
Eluant		0.1 M tris-HCl+1 M NaCl, pH 8.0
Flow rate		2.7 l/h
Elution behaviour		see fig. 10
Capacity		5 l treated serum/24 h

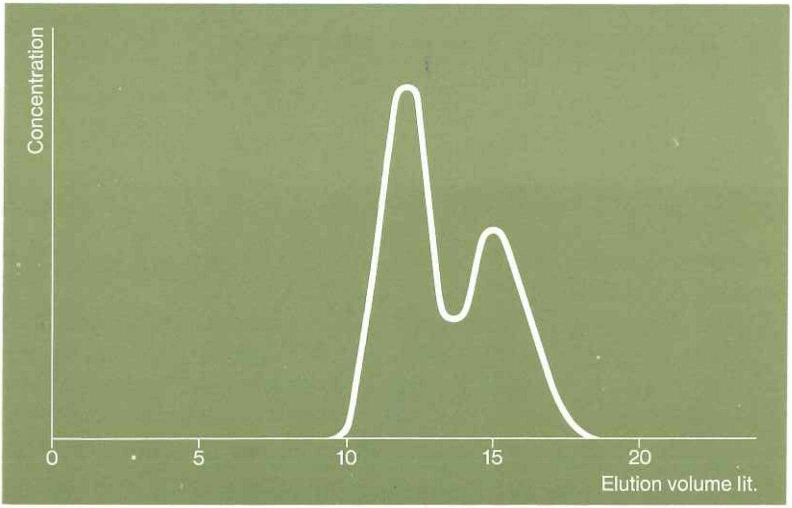

Fig. 10 Fractionation of horse serum proteins on Sephadex G-100 on a production scale. The column used in the experiment permits treatment of 5 l serum/24 hours. The first peak in the elution diagram contains macroglobulins, γ-globulins, haptoglobins etc. The second peak mainly contains the serum albumin, with slight admixtures of other proteins (transferrin etc.). For detailed information on the experiment, see above.

Centrifuge desalting

A very promising method for desalting and separation on a preparative and industrial scale is the basket centrifuge technique. This method has two major advantages: it permits the treatment of viscous solutions and gives practically no dilution.[1790]

The principle of the procedure is as follows:
An annular gel filter cake devoid of liquid in its void space is prepared by centrifugation of a slurry of Sephadex beads in a perforated basket centrifuge lined with a suitable filter medium. A sample is applied at a low centrifugal speed and penetrates the cake without leaving it. After sufficient time for equilibration the desalted sample is spun out and collected. Solutes which have penetrated the Sephadex beads are washed out at a low speed or even at rest. The procedure is applicable for Sephadex G-25 and the still tighter gels G-15 and G-10. For Sephadex G-25 beads the following procedure is recommended with for instance an MSE 3000 basket centrifuge with a cake capacity of 3 litres (MSE, 25—28, Buckingham Gate, London, SW 1).

The perforated basket is lined with a material which is permeable to liquids yet retains the Sephadex beads. A suitable lining is for instance 1/32 inch thick Vyon® (porous polythene) net (Porous Plastic Ltd., Dagenham Dock, England) held in place by expansion rings. The Sephadex slurry is poured into the centrifuge at rest and stirred to a homogenous mixture before starting the centrifuge. The centrifugal field is then quickly raised to 1000×g (2800 rpm). It is kept at that level until no more water is spun out, i.e. until the void volume has left the cake. The Sephadex, at this stage, forms an even layer round the surface of the basket. The speed is lowered to 60×g (700 rpm) and a sample with a volume of 15—20 % of the cake capacity of the centrifuge (450—600 ml) is applied. The speed of the centrifuge is kept at 60×g for about 10 minutes, while the sample progresses through the Sephadex layer. The speed is then increased to 1000×g and kept there until no more solution leaves the centrifuge. The sample, freed from low molecular weight constituents is recovered almost quantitatively. The low molecular weight substances retained in the Sephadex layer can be rinsed out with water at 60×g and the desalting procedure may now be repeated. With coarse grade Sephadex, the retained salt is best washed out with the centrifuge at rest.

For Sephadex G-25 the best results are obtained with the following grades:

Relative sample viscosity (20°C)	Grade
1— 50	Superfine
25— 200	Fine
50— 500	Medium
200—2000	Coarse

Example of centrifuge desalting

Charge solution	volume:	0.50 l
	composition:	191 g Dextran T 250 / l
		48.8 g NaCl / l
	viscosity:	90 cps (20°C)
Output solution	volume:	0.54 l
	composition:	161 g Dextran T 250 / l
		0.07 g NaCl / l
Recovery of dextran		91 %
Desalting efficiency		99.8 %
Cycle time		about 2 h
Capacity		about 1.0 kg desalted dextran / 24 h

Other applications

Concentration

High molecular weight substances can be concentrated by treatment with dry Sephadex G-25 Coarse. Water and low molecular weight substances are taken up by the swelling Sephadex beads, while the high molecular weight substances remain in the external solution. After ten minutes the gel grains are removed from the mixture either by a centrifuge (basket type centrifuge or filter adaptors in centrifuge tubes) or by filtration under pressure. In this way an almost threefold concentration of high molecular weight solutes can be obtained while the ionic strength and the pH are unchanged. The process can be repeated to concentrate the solute even further. This procedure is simple and rapid and is especially useful for very labile proteins.[14, 1132, 1284, 2785]

Partition chromatography with organic solvents

The hydrophilic character and insolubility of Sephadex also make it useful for column partition chromatography, where one phase is an aqueous solution.[605] The aqueous phase is held in the gel as the stationary phase, while the organic solvent, which is unable to enter the gel, becomes the mobile phase. The advantage of Sephadex is that such a large proportion of the bed volume is effective as a stationary phase.

Partition chromatography on Sephadex beds has been used successfully to fractionate antibiotics, transfer RNA, peptides and amino acids. When preparing a bed for partition chromatography, Sephadex should be swollen and packed in the aqueous phase saturated with the organic solvent. The aqueous medium in the void volume is then replaced by the organic phase saturated with water. Lengthy equilibration of the bed with the mobile phase is necessary before sample application.

Zone precipitation

This method is based on the precipitation of a substance which occurs when the concentration of a precipitating agent, e.g. ammonium sulphate, reaches a certain limit.[125] A decreasing concentration gradient of the precipitation agent is introduced into the column. When the concentration of the precipitating agent applied to the column has reached a sufficiently low level, the sample is introduced followed by a non-precipitating eluant.

The high molecular weight components of the sample move faster than the gradient. When they reach a zone sufficiently concentrated in the precipitating agent they come out of solution. As the experiment proceeds, the precipitated substances redissolve and move through the column at the rate of the gradient continuously precipitating and redissolving.

Zone electrophoresis

Sephadex can be used in zone electrophoresis as a supporting material in vertical columns as well as in horizontal troughs and on thin-layer plates. In electrophoresis, Sephadex has the advantage of giving sharp and well-defined zones. The material exhibits low electroosmotic flow and negligible protein adsorption and is also very easy to handle.[122, 337, 467, 710, 1431, 2067]

EXPERIMENTAL TECHNIQUE

General

Preparation of the gel

To obtain satisfactory flow rates through the gel and good separations, the gel must be prepared carefully. The gel should be allowed to swell in excess solvent and left to stand for at least the period of time given in table III.

During swelling, excessive stirring may rupture the beads and should, therefore, be avoided. For all the gel types, the process of swelling can be accelerated by putting the gel slurry on a boiling water bath.

Table III

Type of Sephadex	Minimum swelling time	
	at room temperature	on boiling water bath
G-10, G-15, G-25, G-50	3 hours	1 hour
G-75	24 hours	3 hours
G-100, G-150, G-200	3 days	5 hours
LH-20	3 hours	—

Air bubbles may be trapped in the gel slurry, causing uneven flow when the beds are packed. This problem is avoided by swelling the slurry on a boiling water bath. Alternatively the slurry may be deaerated before packing.

Prevention of microbial growth

Microbial growth rarely occurs in running columns but steps should always be taken to prevent infection of packed columns and gel suspensions. Antimicrobial agents may be eluted from columns before chromatographic runs or they may be present in the eluant during chromatography. Antimicrobial agents which interact with sample substances must be avoided if they are to be used in eluants, otherwise any agent which does not interact with the gel may be used. Some of the more commonly used antimicrobial agents are described below.

Sodium azide (NaN_3), 0.02 %, is very widely used. It does not interact notably with proteins or carbohydrates or change their chromatographic behaviour. Sodium azide interferes with fluorescent marking of proteins, the anthrone reaction and inhibits certain enzymes.

Chloretone (trichlorobutanol), 0.05 %, is effective only in weakly acidic solutions.

Merthiolate® (Thimerosal, ethyl mercuric thiosalicylate), 0.005 %, is most effective in weakly acidic solutions. It is bound to and inactivated by substances containing thiol groups.

Hibitane® (Chlorhexidine), 0.002 %, is a very effective antimicrobial agent. It is incompatible with only a very few substances.

Phenyl mercuric salts (acetate, borate, nitrate), 0.001—0.01 %, are effective only in weakly alkaline solutions.

Chloroform, butanol and toluene are not recommended as antimicrobial agents for Sephadex G-100, G-150 or G-200, as they cause the gel particles to shrink slightly. In addition they are effective only in very concentrated solutions. They penetrate plastic parts of chromatographic equipment, softening the plastic and leaving the liquid without antimicrobial activity. Oxidizing substances should not be used as antimicrobial agents. Autoclaving of the gel, see page 12.

Storage and drying of Sephadex

After use, Sephadex can conveniently be stored in the swollen state (preferably under refrigeration) after sterilization or the addition of an antimicrobial agent. However, once swollen it can be dried and restored to its original state by the following procedure. The swollen gel is thoroughly washed with water to remove salts and contaminants. After removal of excess water, the gel can be shrunk by successive addition of alcohol solutions of increasing percentage alcohol. The gel should be allowed to equilibrate in between each addition. Final shrinking should be with 96 % alcohol. The gel is then sucked dry on a Büchner funnel and finally dried at 60—80°C. A last wash with diethyl ether reduces the drying time. Clumps may appear during the shrinking process but they disperse on re-swelling. Risk for clump formation is reduced by slow shrinking and ether washing.

Gels can also be stored partially shrunk in, for example, 60—70 % alcohol.

Column chromatography

Selection of chromatographic columns

To obtain the best results from gel filtration experiments, care in the choice of column equipment is necessary.

For analytical purposes, a column with an internal diameter of approximately 2.5 cm often proves satisfactory. Narrower columns sometimes tend to give slight wall effects. If the column is too large, dilution becomes a disadvantage.

Preparative gel filtration on a laboratory, pilot-plant or industrial scale should be carried out in suitably designed columns. Such columns, with volumes up to 2.500 litres are available from Pharmacia Fine Chemicals and are described in the booklets "Column K 100/100 and K 215/100" and "Industrial Gel Filtration with the Sephamatic System".

The height of the gel bed to be used depends on the nature of the separation. For desalting purposes short columns may be used. For instance, gel beds of 50 cm length or less can be used with the coarse grade of Sephadex. With the fine grade still shorter beds give satisfactory resolution. For more critical separations, longer beds should be used.

The mixing chambers at the inlet and the outlet should always be kept as small as possible. Many simple columns have large mixing chambers and should be avoided. Bed supports made from coarse sintered glass or porous plastic cannot be recommended because they easily become clogged by small gel particles.

For the convenience of customers, Pharmacia Fine Chemicals has developed a series of standard columns for gel filtration. The design is based on many years' experience in the field of gel filtration. They are manufactured from selected construction materials which do not cause destruction of labile biological substances.

A special column has been developed for experiments with organic solvents, because some parts of the ordinary Sephadex columns are not compatible with these solvents.

The columns have the following special features:

- dead space at the outlet of less than 0.1 % of the column volume. minimizes dilution and prevents remixing

- nylon fabric bed support permits easy and even flow of the liquid as it does not get clogged during the experiments
- columns are easily dismantled and re-assembled
- most of the columns can be equipped with a flow adaptor for upward flow experiments and automatic or semi-automatic sample application.

Packing the Sephadex bed

Correct packing of the bed is of the utmost importance for good performance of a gel filtration column. Irregularities in the packing give rise to uneven flow through the column which often results in zone broadening. This will inevitably give rise to extensive dilution which can lead to mixing of otherwise well separated zones. The flow rates obtainable are affected by the packing technique. If too high an operating pressure is used during packing the gel bed becomes compressed thus causing high resistance to flow.

The column should be mounted vertically and the dead space under the net and in the tubing filled with eluant. This can be done by pouring eluant into the column and sucking away air bubbles under the net with a pipette or by pumping the liquid back and forth through the net with a syringe attached to the outlet tubing. A very simple method is to inject liquid into the outlet tubing and pump it up through the bed support net. When the dead space is properly filled close the outlet tubing.

The gel, previously swollen in eluant (page 31) should be allowed to settle and excess eluant removed until a fairly thick slurry is formed. This slurry should not be so thick that it retains air-bubbles. The slurry should be poured carefully into the column either down the wall of the column or down a glass rod. If the column is not filled with slurry add eluant until the column is completely full. Connect an eluant reservoir to the column and remove the last traces of air through the air vent in the column top piece. An air pocket in the top of the column will cause the eluant to drop into the gel suspension and will thus disturb the even settling of the gel particles. It may not always be possible to fill the column completely with gel at one time. In such cases an extension tube should be mounted on top of the column. A Gel and Eluant Reservoir has been specially designed for this purpose by Pharmacia Fine Chemicals. Pour all the gel required for the experiment into the column and the gel reservoir and start the flow immediately. Preparing the gel bed from a thin suspension or packing the column in stages often results in a badly packed bed.

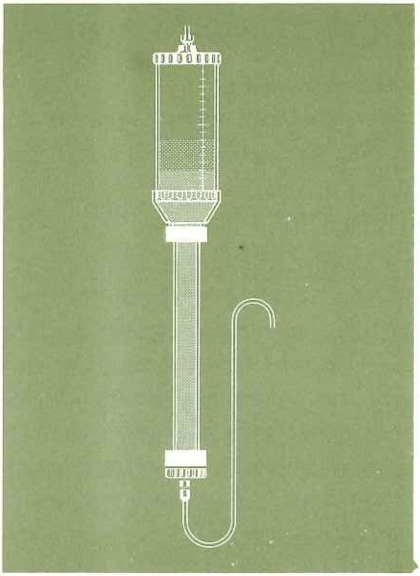

Fig. 11 Packing a column with the aid of a Gel and Eluant Reservoir converted into an extension tube (gel reservoir). When all the gel has settled into the column the reservoir is removed, converted into a Mariotte flask and used for equilibration and elution.

The flow should be started as soon as possible after filling the column, in order to obtain an even sedimentation. When the column has been filled a Gel and Eluant Reservoir converted to a Mariotte flask can be connected to the column. The operating pressure must be checked before opening the column outlet tubing. The operating pressure depends on the difference between the free surface of eluant in the reservoir and the outlet and is illustrated for different set-ups in Fig. 12. With a Mariotte flask the operating pressure is measured from the end of the air inlet tube. With the soft gels Sephadex G-75 to G-200 the operating pressure must not exceed certain limits which are given in table V.

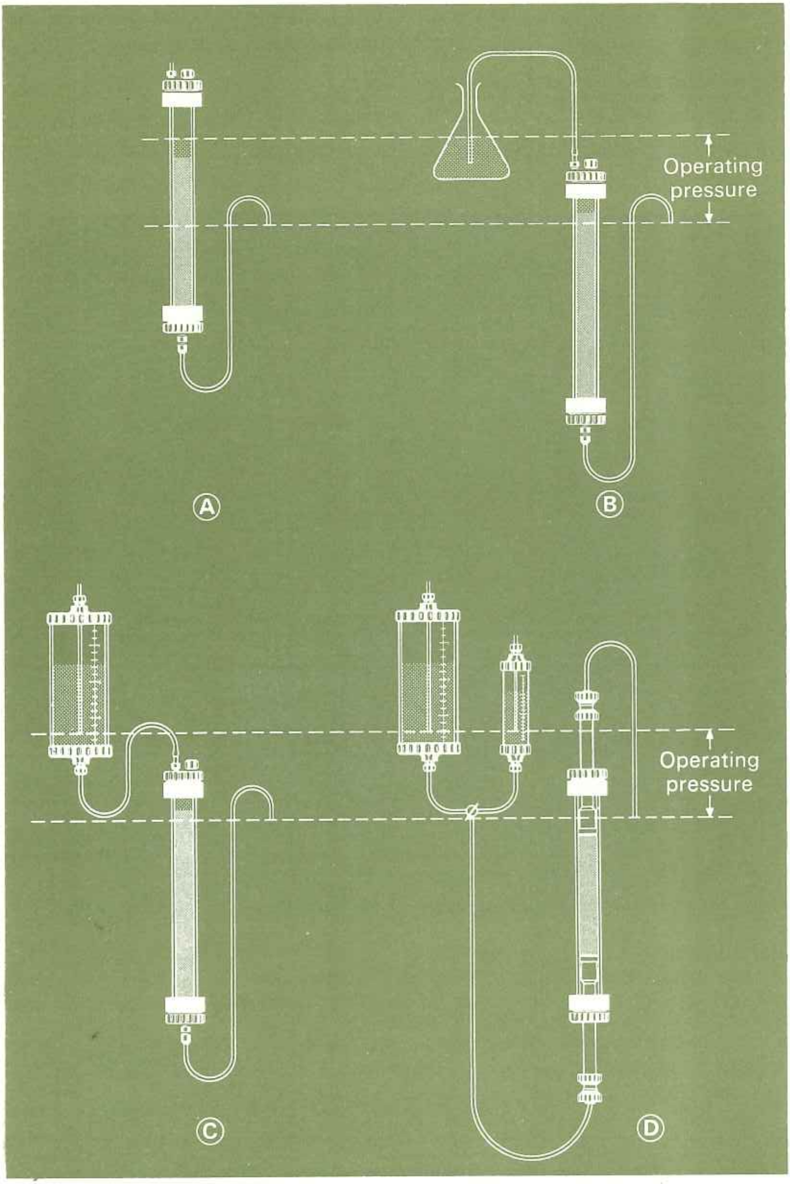

Fig. 12 Definition of operating pressure.
A and B. Pressure is measured between the free eluant surface in the column or the reservoir and the end of the outlet tubing.
C and D. Pressure is measured from the bottom of the air inlet tube in the Mariotte flask to the end of the outlet tubing, no matter whether the flow is downward (C) or upward (D).

Two to three column volumes of eluant should be passed through the column in order to stabilize and equilibrate the gel bed. The same or slightly higher flow rate to be used in the experiments should be used. If the column is to be left unattended a safety loop arrangement will prevent the column from running dry (see fig. 13).

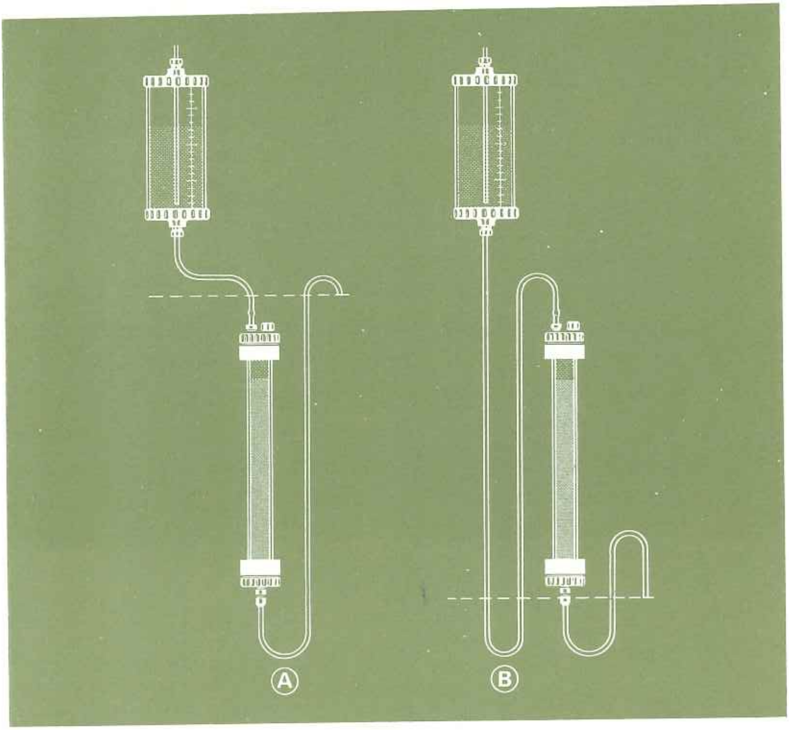

Fig. 13 Safety loop arrangements.
A. The safety loop is placed after the column and the end of the outlet tubing is placed above the column. The flow stops when the eluant in the inlet tubing reaches the level of the outlet tubing.
B. The safety loop is placed before the column with the column outlet tubing in any position above the lower loop on the inlet side. The flow stops when the eluant in the inlet tubing reaches the level of the outlet tubing.

Before starting any experiments it is advisable to check the homogeneity of the bed by running through a solution of a coloured substance. Blue Dextran 2000 (Pharmacia Fine Chemicals) at a concentration of 2 mg/ml can be used for this purpose. The quality of the packing can be checked by watching the progress of a zone of this substance through the bed. Visual inspection of the bed in transmitted light may also reveal heterogeneities and air-bubbles.

Packing Sephadex LH-20. In most solvents the same method can be used for packing beds of Sephadex LH-20 as for packing beds of Sephadex G-types. In some solvents, notably chloroform, Sephadex LH-20 is lighter than the solvent and floats in it. A column equipped with two flow adaptors, i.e. adjustable end pieces, is recommended for gel filtration in organic solvents of low specific gravity. Pour all the required slurry into the column and drain until there is space enough to insert the top adaptor. Having locked this adaptor in position at the surface of the gel, the chloroform flow is directed upwards. The bed will now be packed against the upper flow adaptor and it can be fixed in that position by slowly pushing the lower adaptor upwards.

Bed surface protection. Before sample application, the upper surface of the gel bed should be protected with a piece of suitable material, e.g. filter paper, porous plastic or nylon net. With some types of columns (Pharmacia Fine Chemicals) a sample applicator is delivered. This is a nylon net mounted on a small acrylic plastic cylinder. Care should always be taken not to trap air under the bed surface protection. The easiest way of inserting the sample applicator is as follows. Clean it thoroughly before use and then dry the net by pressing the sample applicator against a dry cloth or tissue. With the column almost filled with liquid insert the sample applicator at a slight angle.

Sample application

Sample application on drained bed surface. Most of the eluant above the gel surface is removed by suction. The column outlet is opened and the remaining eluant drained away. After closing the outlet the sample is carefully layered on top of the bed. The column outlet is then opened. After the sample has drained into the bed, the gel surface and the column wall in contact with the sample are washed with a small amount of eluant. Under no circumstances should the bed run dry during this procedure. The column is then filled with eluant and connected to a Mariotte flask.

Sample application under the eluant. A sharp zone can be obtained if the sample is layered on top of the gel bed, underneath the eluant. The sample must be denser than the eluant. If it is not, it can be made so by the addition of a small amount of, for instance, glucose, sodium chloride, buffer salt or another suitable inert material. Layering can be done with a pipette with a bent tip or a syringe fitted with a piece of fine capillary tubing. The sample is sucked into the syringe and a small air bubble formed at the outlet of the tubing. This tubing is put into the eluant and the end held a few mm above the bed surface. The sample is squeezed out at an even rate. At the end of the application eluant is drawn into the capillary to prevent sample mixing with eluant when the tubing is withdrawn. No rinsing is required after sample application since the sample will be quantitatively transported into the gel bed by the eluant.

Sample application with a flow adaptor. A very convenient way of applying samples is to use a flow adaptor, i.e. an adjustable column end piece. This is the only possible method in upward flow elution. Flow adaptors are available for most column types from Pharmacia Fine Chemicals and should be used in the following way.

The adaptor mechanism is loosened and, with the column nearly full of liquid, it is inserted, at an angle, into the column. Air bubbles must not be trapped under the net. The adaptor should then be tightened in the column and pressed downwards until it is in contact with the bed surface. The eluant above the bed will leave through the capillary tubing of the adaptor. It is important to have the column outlet tubing closed when the adaptor is inserted. If the outlet is left open the gel bed will become compressed. Samples can then be applied through the adaptor either by putting the adaptor tubing in the sample reservoir or by connecting this tubing to eluant and sample reservoirs via a three-way valve.

Sample size

For analytical purposes where the main objective is to obtain as good a separation as possible the starting zone should be narrow. This is accomplished by using a small sample. Using a sample of less than 1—2 % of the bed volume, however, does not improve the separation result.

On a preparative scale, where the objective is to obtain a maximum yield with a given degree of separation, the sample should be made as large as possible within the limits set by the requirements of the separation. The volume which separates the front of the elution curve of one substance from that of another substance, that is the difference between their elution volumes, is called the separation volume:

$$V_{sep} = V'_e - V''_e = V_s(K' - K'') \quad (1)$$

(see page 56).

Were no zone broadening to take place, the whole separation volume could be filled between the substances eluted most closely together. The sample size in this theoretical case could then be as large as the separation volume. However, due to microturbulence, non-equilibrium between the stationary phase and the mobile phase and longitudinal diffusion in the bed, the zones will always be broadened. The sample size must therefore be smaller than the separation volume. This is illustrated in fig. 14. Fig. 6 gives an example of the elution curves in desalting operations on Sephadex G-25 at different sample volumes. This figure also shows that a larger sample volume gives a smaller dilution. As much as 25—40 % of the bed volume can be applied.

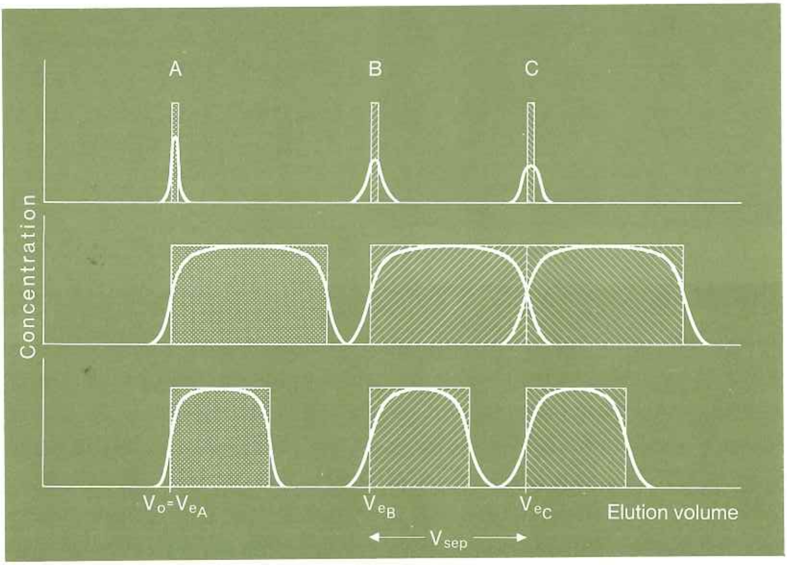

Fig. 14 Elution curves for different sample size. The top diagram corresponds to the application of a small sample. The centre diagram corresponds to the maximum sample size giving complete separation if no zone broadening were to take place. The bottom diagram corresponds to the maximum sample volume to be applied to obtain complete separation in the conditions of the experiment. The shaded areas correspond to the elution profiles that would be obtained if no zone broadening were to take place.

Sample composition

Due to the linear partition isotherms, gel filtration is remarkably independent of sample concentration. However, in addition to the solubility of the solutes, the viscosity of the sample often limits the concentration that can be used. A high viscosity of the sample causes instability of the zone and an irregular flow pattern. This leads to very broad and skew zones. The critical variable is the viscosity of the sample relative to the eluant. This is illustrated in fig. 15, giving the elution profiles of haemoglobin and NaCl at different sample viscosities.

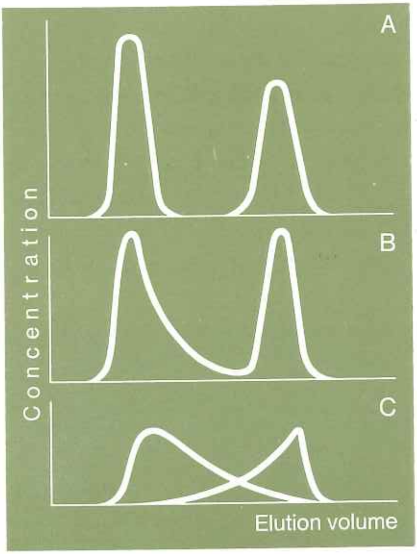

Fig. 15 Elution diagrams obtained when haemoglobin and NaCl were separated. Experimental conditions were identical except that the viscosities were altered by the addition of increasing amounts of dextran. An increasing deterioration of the separation becomes strikingly apparent. (A lower flow rate will not improve the separation.)

Experiment	Dextran T 250 %	η rel
A	0	1.0
B	2.5	4.2
C	5.0	11.8

Eluant

Completely uncharged substances can be eluted with distilled water. When chromatographing substances carrying charged groups, an eluant with an ionic strength of at least 0.02 gives better results, as Sephadex contains a small number of carboxyl groups giving rise to an adsorption of small amounts of cations and an exclusion of small amounts of anions. If the product is to be lyophilized, a volatile buffer such as ammonium acetate or ethylenediamine acetate can be used. In desalting, the separation volume is so large that in general charged substances can also be treated with distilled water as eluant. A complete removal of salt is not possible, but the amount of ions excluded, and therefore eluted in the high molecular weight fraction, is so small that it can be neglected in most cases.

Elution

The flow of liquid in the elution step can be produced by difference in hydrostatic pressure or by a pump.

When the flow is maintained by gravity feed a constant pressure flask, such as the Gel and Eluant Reservoir converted as a Mariotte flask is recommended for this purpose. Note that in the case of a Mariotte flask, the operating pressure corresponds to the vertical distance between the lower end of the flask air inlet tube and the free end of the column outlet tubing. The experimental set-up with Mariotte flasks attached is illustrated in fig. 12.

Flow rates

It is not possible to apply one set of rules for calculating flow rates over the whole range of Sephadex types. Sephadex G-10, G-15, G-25 and G-50 obey one set of rules whilst Sephadex G-75, G-100, G-150 and G-200 follow another set. The arguments, equations and data given here apply only to laboratory columns with diameters up to 5 cm.

Flow rates in columns packed with Sephadex G-10, G-15, G-25 and G-50

These gels may be assumed to behave as rigid spheres in gel filtration and therefore obey Darcy's Law, i.e.

$$U = K\frac{\Delta p}{L} \qquad (2)$$

where

U = linear flow rate expressed in cm/h (ml/cm^2.h)

Δp = pressure drop over gel bed expressed in cm H_2O

L = bed height expressed in cm

K is a constant of proportionality depending on the properties of the bed material and the eluant.

Assuming an eluant with viscosity = 1 cp one can write

$$U = K_o\frac{\Delta p}{L} \qquad (3)$$

where K_o is the "specific permeability" dependent on the particle size of the gel beads and their water regain. Observe that the flow rate is proportional to the pressure drop over the bed and, assuming a constant pressure head, inversely proportional to the bed height. This means that *maximum flow rates will never be obtained in laboratory columns since maximum operating pressure cannot be reached.* Notice that (to a good approximation) the flow rates are independent of the column diameter.

Flow rates at viscosities greater than 1 cp can be obtained by using the relation: flow rate inversely proportional to viscosity. At first sight, it would appear that high eluant viscosities lead to poor flow rates but the operating pressure can be increased to compensate for the viscosity effect. Temperature has no direct effect on the flow rate but, of course influences the viscosity of the eluant. Slightly lower flow rates are obtainable, therefore, in a cold room than at room temperature.

Theoretical flow rates (not maximum) can be calculated from equation (3) by inserting values for Δ p and L. Specific permeabilities are given in table IV.

Table IV

Sephadex type	Permeability K_o
G-10	21
G-15	22
G-25 Superfine	9
Fine	25
Medium	94
Coarse	260
G-50 Superfine	13.5
Fine	36
Medium	145
Coarse	400

Flow rates in columns packed with Sephadex G-75, G-100, G-150 and G-200

Calculation of flow rates in beds of Sephadex with high water regain is somewhat more complicated since Darcy's Law is not applicable. Not only is the flow rate dependent on the factors already mentioned but also on the column diameter. For these gels *a maximum flow rate is reached at an optimal operating pressure.*

Flow rates together with the operating pressures are given for 1.5 cm, 2.5 cm and 5 cm columns in table V. A bed height of 100 cm has been assumed. To calculate the flow rate for a different bed height the flow rate may be assumed to be inversely proportional to the bed height.

Table V

Maximum flow rates for G-75, G-100, G-150 and G-200 at optimal operating pressures

Gel type	Column diameter 1.5 cm			Column diameter 2.5 cm			Column diameter 5.0 cm		
	Operating pressure	Flow rate		Operating pressure	Flow rate		Operating pressure	Flow rate	
	cm H_2O	ml/cm^2.h	ml/min	cm H_2O	ml/cm^2.h	ml/min	cm H_2O	ml/cm^2.h	ml/min
G-75	50—200	25	0.74	40—160	23	1.9	38—150	18	5.9
G-75 Superfine	50—200	6	0.18	40—160	5.5	0.45	38—150	4.5	1.5
G-100	25—100	16	0.47	24—96	15	1.2	19—76	12	3.9
G-100 Superfine	25—100	4	0.12	24—96	3.7	0.3	19—76	3	1
G-150	10—40	7.4	0.22	9—36	7	0.57	8—32	5.5	1.8
G-150 Superfine	10—40	1.8	0.05	9—36	1.7	0.14	8—32	1.4	0.45
G-200	5—20	4	0.12	4—16	3.6	0.3	3—12	3	1
G-200 Superfine	5—20	1	0.03	4—16	0.9	0.07	3—12	0.8	0.26

The flow rates in table V have been calculated assuming a viscosity of 1 cp and a dry particle diameter (harmonic mean) of 30 μ for superfine grades and 60 μ for the ordinary grades. Note that the operating pressure is the pressure drop over the bed and therefore takes no account of the reduction in pressure caused by tubings and connections in an experiment.

Errors in the flow rate figures, given in table V, may be as much as 30 % since the calculations are dependent on other factors not taken into account, such as the packing of the bed and temperature. Nonetheless the flow rate table should serve as a useful guide to the flow rates obtainable on Sephadex types with high water regain.

Influence of flow rate and particle size on zone broadening

High flow rates and coarse grade bed material invariably give large zone broadening especially in small columns. For analytical purposes the superfine or fine grade material should thus be used, and the flow rate should be kept as low as convenience permits.

For preparative purposes the use of a higher flow rate (and consequently a faster separation) often outweighs the loss of resolution in the chromatographic run. The influence of flow rate and particle size are illustrated in figs 16 and 17.

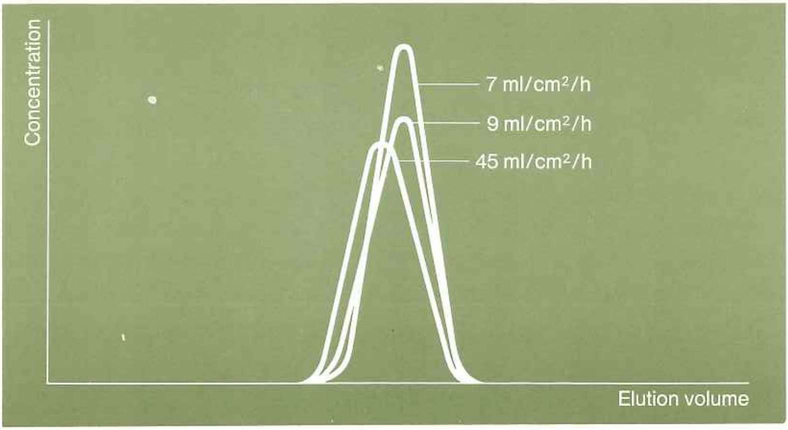

Fig. 16 Elution diagrams of glucose on Sephadex G-25 at different flow rates. A slight displacement of the peak is observed, indicating that a complete diffusion eqilibrium has not been reached.

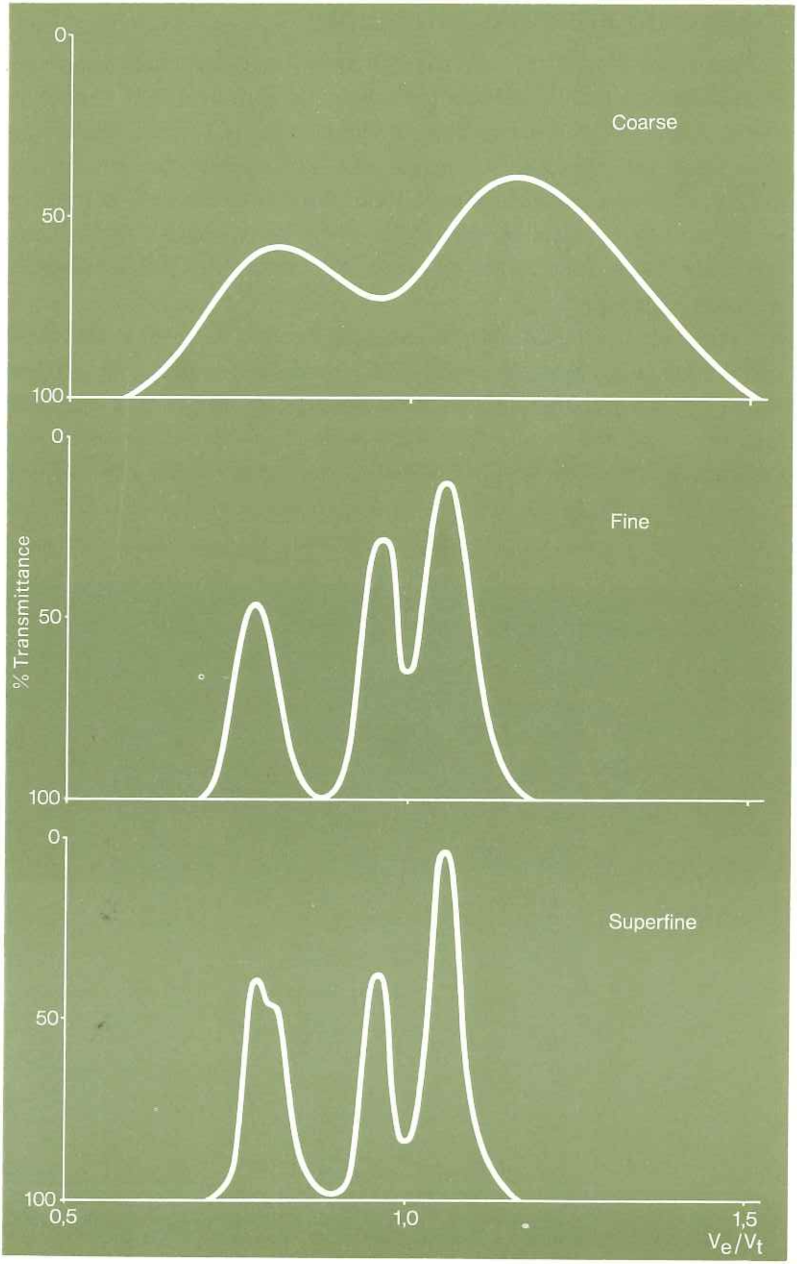

Fig. 17 Gel filtration of cytidylic acid, cytidine and cytosine on Sephadex G-25 of different grades. On the coarse grade only cytosine and cytidylic acid were chromatographed.
Bed diameter: 2.4 cm. Bed volume: appr. 200 ml. Flow rate: appr. 1 ml/min. Eluant: 0.025 M phosphate buffer, pH 7.0.

Increasing effective bed height

If incomplete separation is achieved between two substances due to an insufficient separation volume (see page 41) a longer bed should be used. If this involves inconveniently long beds and impractically low flow rates, the effective bed height can be increased by arranging a battery of several columns. The outlet of the first is attached to the inlet of the next by capillary tubing. If the connections between the columns are made using flow adaptors, zone broadening in the connections becomes negligible.

An increase in effective length can also be achieved by a technique called recycling chromatography. This involves pumping the effluent from the outlet of the column back to the top. Peaks that are satisfactorily separated in the first cycle, should be eluted, as there is a definite risk that after a number of cycles these peaks will overtake

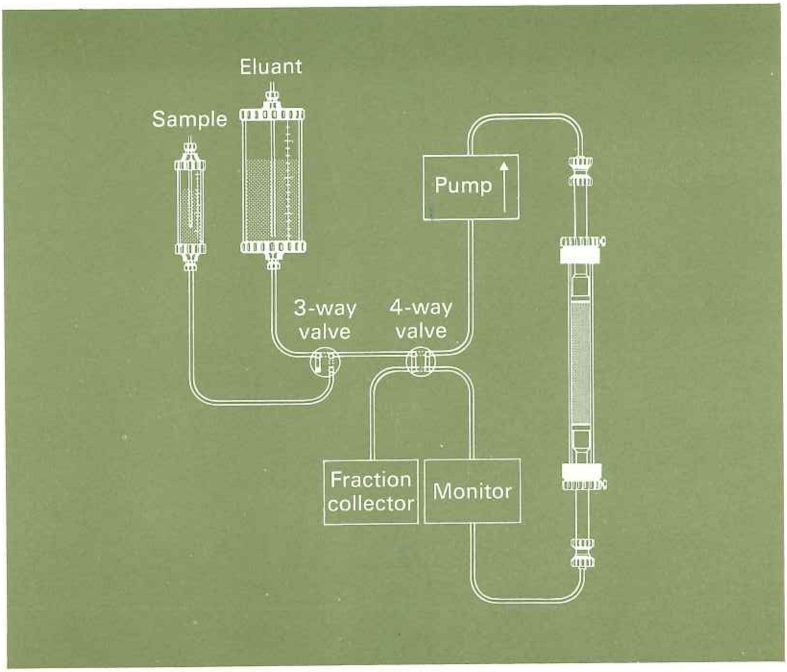

Fig. 18 Increasing the effective column length.
A set-up for recycling gel filtration using a three-way and a four-way valve. Eluant and sample are connected to the three-way valve which can also be closed during the recycling. The four-way valve connects the column outlet to the inlet or sample/eluant to the column and the column outlet to a fraction collector.

other peaks and remixing will occur. Long beds should be used to decrease zone broadening caused by pumping and to reduce the risk of remixing. The use of long beds also reduces the number of cycles required to achieve complete separation of the substances under study.

Repacking

After an experiment no special regeneration of the bed is necessary. The flow rate may be somewhat reduced after lengthy use, mainly because of compression of the gel bed under the influence of the flow. Original flow rates can be restored by backwashing, repacking or reversal of the flow.

Very often a reduction of the flow rate is caused by dust particles etc., accumulating on top of the gel bed. This can be remedied by stirring up a few centimetres and allowing the gel to settle again or by removing a thin layer from the surface of the bed.

Thin-layer gel filtration

In the last few years the technique of thin-layer chromatography has expanded rapidly and it is now used in many different fields. Thin-layer gel filtration has many advantages over the column technique but there are also disadvantages. Thin-layer gel filtration is a simple, rapid, microanalytical technique permitting the elution of many samples on one plate. However, it is a method which is difficult to quantitate and determination of separated substances can be difficult as no fractions, on which tests can be made, are collected.

For thin-layer gel filtration the superfine grade of Sephadex should be used. The preparation of the material is the same as for column chromatography. Sufficient swelling time should be allowed. After swelling, the gel particles are allowed to settle, preferably over night, and the supernatant is then removed by suction or by decantation. With the gels G-25—G-50, a small amount of liquid is left above the gel surface.

Spreading

The glass plates, which can be of any size compatible with the chromatographic equipment, must be thoroughly cleaned by washing with a detergent and distilled water or by storing the plates in a concentrated solution of sodium carbonate. This should be followed by thorough washing with water before use. Any traces of dirt or fatty materials remaining on the surface of the plates will result in unsatisfactory spreading of the Sephadex.

The clean dry plates are then coated with the swollen gel. Any of the commercially available spreading devices may be used. More satisfactory results can be obtained by using a rod, which can be moved back and forth over the glass plate. It is kept at a suitable distance from the plate by collars (e.g. consisting of electrical tape) at its ends or by distance pieces positioned along the edges of the glass plate. The gel adheres instantly to the plates without the need for added fixative. In the literature the use of layers of different thicknesses has been reported. Layers of 0.5—1.0 mm usually give the best results.

Set-up and experimental technique

The plates should be run by descending chromatography in a closed chamber, without previous drying. An example of an experimental set-up is illustrated in fig. 19. Paper and thin-layer electrophoresis apparatus can often be adapted for this purpose. The bridges carrying eluant to and from the plate should be of thick filter paper (e.g. Whatman 3 MM) in order to have sufficient capacity. Note that the filter paper should be covered to avoid evaporation. The flow rate through the gel is regulated by the angle of the plate to the horizontal. An angle between 10° and 20° has been found to be the most suitable, although other angles have been used.

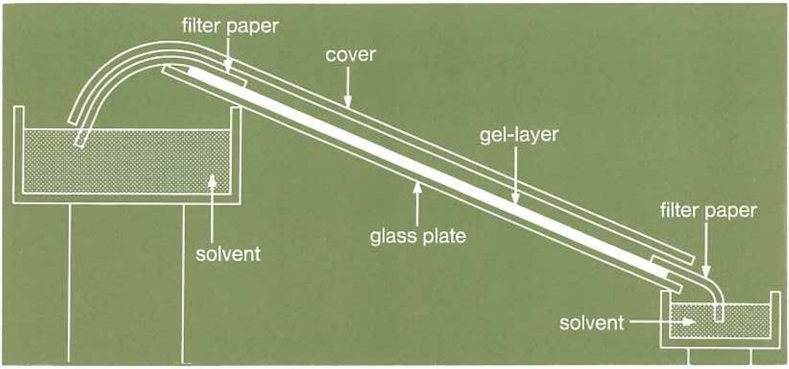

Fig. 19 Experimental setup in thin-layer gel filtration.

Equilibration and sample application. Before a run is started, the plates should be equilibrated by allowing the solvent to flow through the gel for 10 to 15 hours, a step which can easily be carried out overnight. After equilibration, the sample (2—5 μl) is applied through a slit in the cover of the thin-layer apparatus with a micropipette or micrometer syringe as a round spot 2—4 mm in diameter. Sample application is carried out with the plate horizontal and with care not to disturb the gel layer.

Measurement of flow rate. The flow rate through the plate can be measured by the use of a coloured reference substance. Different substances have been used as markers, e.g. coloured proteins such as haemoglobin, cytochrome c, myoglobin or fluorescein isothiocyanate-conjugated serum proteins. The latter have proved very satisfactory and can easily be detected on the plates by their pronounced greenish yellow fluorescence in ultra violet light. If the marker substance does not move with the front its R_F value can be determined in a separate experiment.

The best resolution in thin-layer gel filtration is usually obtained at flow rates not exceeding 4 cm/hour. An experiment on a 40 cm long plate thus requires no attention for 8—10 hours.

Transfer of spots to filter paper and staining. After completion of a run, the separated substances are transferred from the gel layer by absorption into a superimposed sheet of thick filter paper (e.g. Whatman 3 MM). This paper is rolled onto the plate, starting at one end and moving evenly to the other. Air bubbles should not be trapped between the paper and the gel layer with this procedure. After about one minute the sheet is removed and can then be dried and stained according to conventional methods. Extremely small quantities of enzyme can be detected by absorbing the spots into a sheet of paper impregnated with specific substrate.

Alternatively, the spots may be stained directly on the plate after very careful drying of the Sephadex layer. However, this technique cannot be generally recommended because the gel layer has a tendency to crack or pulverize on drying.

Chromatography and electrophoresis

Thin-layer gel filtration can be combined with electrophoresis for better analysis of complex mixtures. Fig. 20 shows a separation of pathological human serum (multiple myeloma serum γ_{1A}-type) on Sephadex G-200 by gel filtration in one dimension followed by electrophoresis in the other dimension.

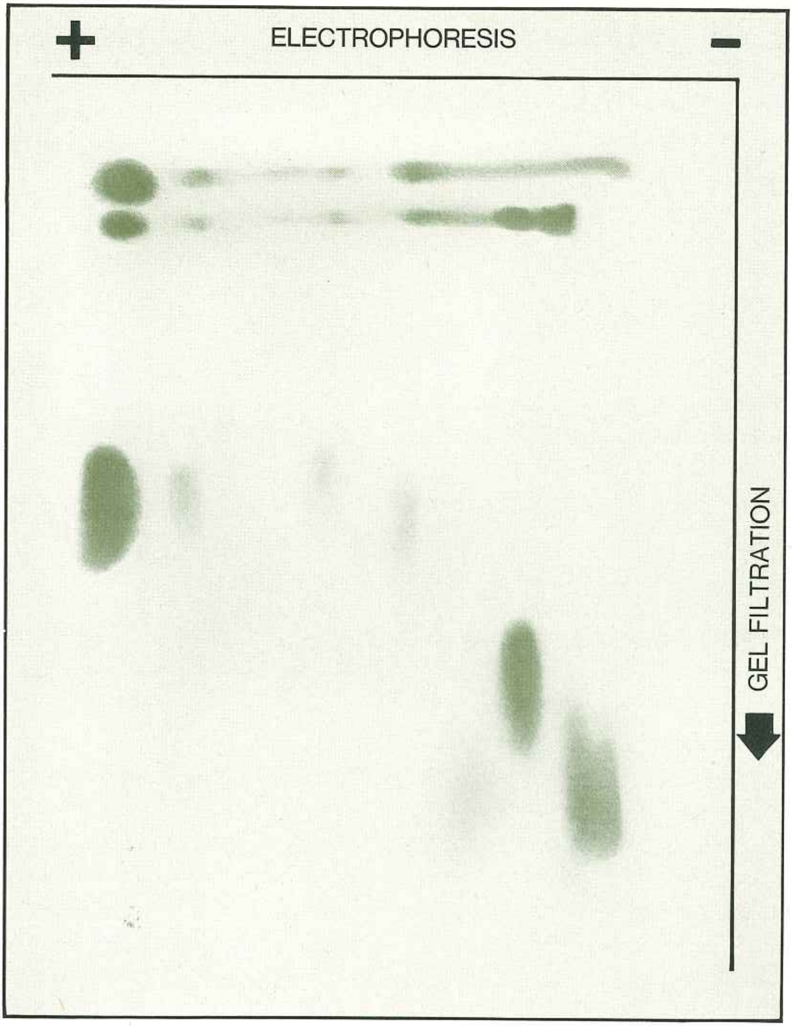

Fig. 20 Gel filtration—electrophoresis.
The figure shows a separation of pathologic human serum (multiple myeloma serum of γ_{1A}-type) on Sephadex G-200 by gel filtration in one dimension followed by electrophoresis in the other dimension. Normal serum and pathologic serum were added as references before electrophoresis was started. The electrophoretic separation was carried out in 0.05 M veronal buffer, pH 8.4, with a potential gradient of 6 V/cm for 6 h. (Hanson, L. Å., Johansson, B. G., Rymo, L., Clin. chim. Acta 14 (1966) 391—398. Reproduced by kind permission of the Authors.)

CALCULATION OF EXPERIMENTAL RESULTS

To characterize the behaviour of a solute in gel filtration, its elution volume (V_e) is measured. Different criteria are used for the determination of the elution volume.

- When very small samples are applied (small enough to be neglected compared with the elution volume), the position of the maximum of the peak in the elution diagram should be taken as the elution volume.
- When samples, whose volumes cannot be neglected compared with elution volume, are applied the elution volume is measured from after half of the sample volume to the position of the maximum of the elution peak.
- When large samples are used (giving a plateau region in the elution curve), the volume eluted from the start of sample application to the inflection point (or the half height) of the rising part of the elution peak should be taken as the elution volume. This criterion is also often somewhat incorrectly applied to samples not giving plateau regions.

These three cases are illustrated in fig. 21.

More sophisticated criteria may also be used to characterize V_e. These, however, involve such complex mathematical calculations that in practice they are of less value.

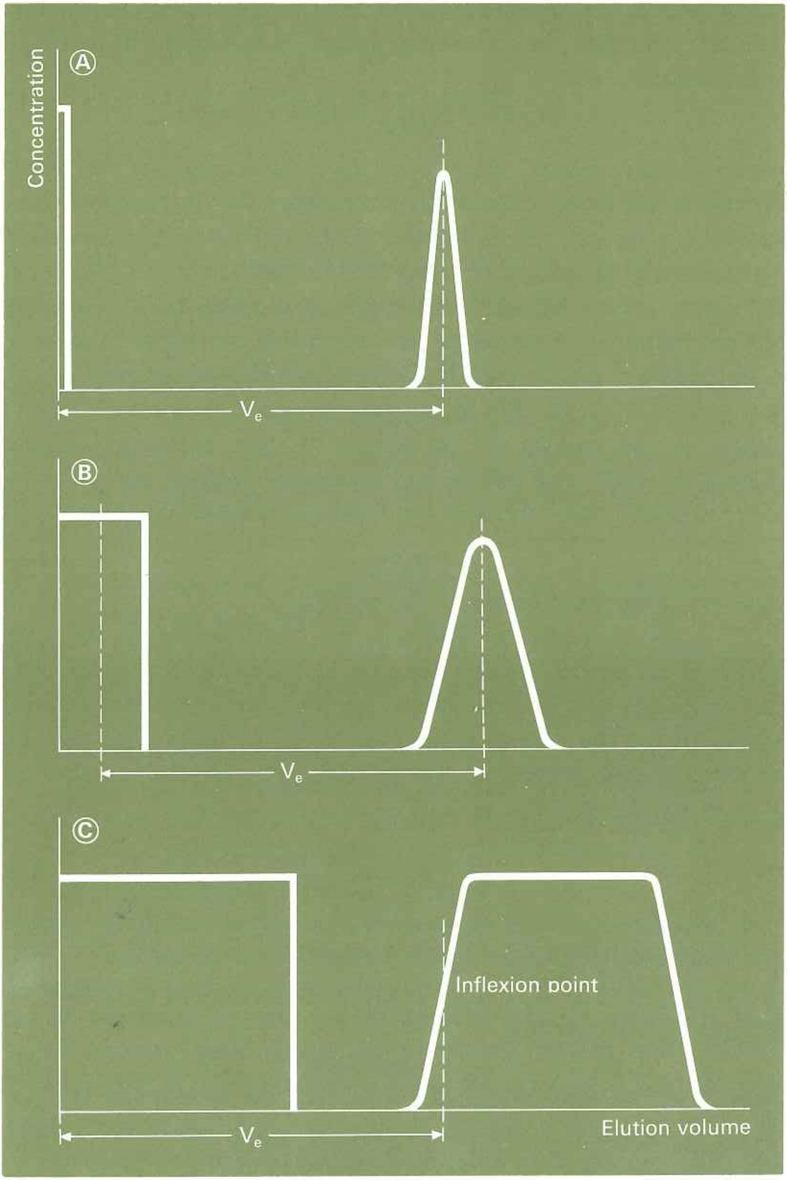

Fig. 21 Measurement of elution volume, V_e.
A. Sample size negligible compared with bed volume.
B. Sample size not negligible compared with bed volume.
C. Sample giving plateau elution curve.

To characterize the behaviour of the solute without reference to the geometric dimensions of the column, the following three variables are most commonly used:

- $$V_e/V_o$$

relative elution volume, where V_o, the void volume (or the dead volume), is the elution volume for a substance that is completely excluded from the gel. V_o is identical to the volume of the interstitial liquid between the gel grains in the bed. In some cases the inverse of the relative elution volume, R, the retention constant, is also used for the characterization of substances. R is interesting because of its relation to the relative migration rate used in thin-layer chromatography.

The value of V_e/V_o is easy to calculate. However, it is somewhat dependent on the packing of the column. A high operating pressure resulting in small void volume may sometimes increase the value of this parameter. These variations are, however, of minor importance.

- $$V_e/V_t$$

where V_t is the total volume of the gel bed.

V_e/V_t is also very easy to determine. The measurement of the total volume of the gel bed is preferably done by filling the chromatographic tube to the original level of the gel bed with water and measuring its volume. It has the same draw-back as V_e/V_o in being susceptible to variations in the packing of the gel bed, although the variation in V_e/V_t is smaller than that in V_e/V_o.

- $$K_{av}=\frac{V_e-V_o}{V_t-V_o} \tag{4}$$

is the partition coefficient between the liquid phase and the gel phase. This variable is independent of the compaction of the gel bed.

In the calculation of K_{av} use is made of the fundamental equation of partition chromatography,

$$V_e=V_o+K\cdot V_s \tag{5}$$

where K is the partition coefficient between the mobile and the stationary phase and V_s is the volume of the stationary phase. For calculation of K_{av} the whole gel phase is considered to be V_s.

THEORETICAL CONSIDERATIONS

The following is an outline of the theories of gel filtration and makes no attempts to cover general chromatographic theory.

In chromatography, the solute is carried through the chromatographic bed by the mobile phase. The stationary phase acts to retard the progress of the solute through the bed. Different solutes are retarded to varying extents. In partition chromatography, this retardation is effected by partitioning the solute between the mobile phase and the stationary phase.

The rate at which a solute passes through the bed is proportional to the part of the time, calculated on a statistical basis, that the molecules spend in the mobile phase. This fraction of the time spent in the mobile phase in turn is determined by the ratio of the volumes of the mobile phase and the stationary phase, and by the partition coefficient. The partition coefficient of a solute between the stationary phase and the mobile phase thus governs its chromatographic behaviour, as is evident from the fundamental equation of partition chromatography (5). Various theories have been advanced to account for the value of this partition coefficient in gel filtration.

The first theoretical approach to the specific behaviour of gels in chromatography is that of *Flodin*.[109] He considers the partition of the solute molecules between the gel particles and the interstitial liquid as an entirely steric effect. He points out that in the immediate environment of the cross-links between the gel chains, the gel matrix occupies a great deal of space. Large molecules cannot penetrate into these regions, while small molecules can approach the cross-links more closely. These steric restrictions, according to Flodin, divide the gel into permitted and forbidden regions. The larger the molecular dimensions of the solute, the greater the proportion of the gel constituting the forbidden region. In the permitted region, the concentration of solute is assumed to be identical to that in the interstitial liquid. This theory leads to the definition of K as the volume available to a given molecular species.

The steric approach to gel filtration has been extended in various ways. According to *Porath*[298], the molecular weight dependence of the partition coefficients in gel filtration corresponds to that which would be produced by conically shaped pores. *Squire*[615] has derived an expression for the molecular weight dependence based on the assumption that the distribution of the pore size would correspond to that produced by a set of cones, cylinders and crevices.

A very interesting approach has been suggested by *Laurent* and *Killander*.[623] They describe the gel matrix as randomly distributed straight rods of infinite length. From this model they calculate the volume available for molecules represented by spheres of various size.

The sterical approach does not take into consideration other factors influencing the distribution between the gel phase and the mobile phase, such as the difference in affinity of the solute to the solvent and to the gel matrix. Partition coefficients with values higher than would be expected from the theories outlined above are described as "adsorption effects". This terminology has the disadvantage of giving the impression that the substances which show "adsorption effects" have non-linear partition isotherms. However, the narrow symmetric elution curves of even very strongly retarded substances indicate that the partition isotherms for these substances are linear.[1271]

Especially for the gels of low water regain, which have a high content of gel matrix, the steric approach is unsatisfactory, because on these gels the effects of differences in affinity (making up for the enthalpy part of the partition coefficient) are often just as great as the steric effects (essentially producing the entropy part of the partition coefficient). This has been shown quite clearly by *Marsden*[830] who calculated the enthalpy and entropy contribution to the K-values of low molecular weight solutes in work with Sephadex G-10 and G-15.

The treatment of gel filtration as a kind of partition chromatography makes a unified treatment possible both for the substances that behave as one would expect from the sterical theories and for those with a behaviour differing from that. If the K-values are not regarded as fractions of the space in the gel but as partition coefficients, K-values higher than 1 are no longer an anomaly. This is even more justified as the partition isotherms are linear both for substances with high and low K-values.

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SELECTED REFERENCES FOR FURTHER READING

The references given in the text of this booklet and in this section have been chosen from the many hundreds of articles published which describe the theory and methodology of gel filtration and the applications of Sephadex. Information on specific studies can be found in the "Literature Reference List" available separately from Pharmacia Fine Chemicals. The number in front of each reference refers to this list.

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